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Change in Body Mass Can Overrule the Effects of Maternal Testosterone on Primary Offspring Sex Ratio of First Eggs in Homing Pigeons

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ABSTRACT

The phenomenon of primary offspring sex ratio adjustment is being extensively studied, yet knowledge of the underlying proximate mechanism is still mainly hypothetical. Female birds are the heterogametic sex, thus potentially controlling the sex of the gamete to be fertilized. In several bird species, independent studies showed effects of maternal plasma testosterone, corticosterone, or condition on primary offspring sex ratio. Our objective was to investigate the causal relation between these two maternal hormones, body condition, and offspring sex ratio in homing pigeons (*Columba livia domestica*). Following our earlier study, we again implanted females with testosterone and determined embryo sex of first eggs. To identify the pathway of sex ratio adjustment, we repeatedly measured not only maternal plasma testosterone and mass but also plasma corticosterone, cholesterol, and glucose, all indicators of body condition. We also calculated the temporal change in these parameters, which has been proposed to be a more accurate predictor of offspring sex ratio compared with the absolute values. Furthermore, we analyzed testosterone concentrations in outer yolk layers, which potentially influence the first meiotic division of the gamete. We found no relation between plasma parameter and embryo sex of first eggs; testosterone treatment did not affect any of the measured parameters. However, females that increased in mass produced more male embryos, irrespective of treatment group. Outer yolk layer testosterone concentrations did not differ between treatment groups or between male and female eggs. We propose that not only the absolute values but also the complex interactions between maternal hormones

and body condition ultimately affect the mechanism of primary offspring sex manipulation.

Introduction

The phenomenon of avian primary offspring sex ratio adjustment to environmental factors has been convincingly demonstrated (reviewed in Pike and Petrie 2003; Alonso-Alvarez 2006). Identifying the proximate mechanisms underlying embryo sex determination and manipulation is crucial for further progress in understanding their function and evolution. Only then can we reveal limitations to the degree of flexibility of offspring sex adjustment and possible trade-offs between the benefits and costs the mother infers from producing the desired sex. Moreover, finding the key to control offspring sex ratio would provide a highly valuable tool from which conservation measures (Robertson et al. 2006), as well as commercial breeding, would greatly benefit.

Trivers and Willard (1973) predicted that mothers bias offspring sex ratio in relation to their own condition and ability to invest in rearing young. Usually, reproductive success of the male sex is more quality dependent than it is in females. Therefore, a son in good condition would provide a higher fitness return than a daughter under comparable circumstances, whereas a daughter in poor condition would most likely return higher fitness benefits than a son. Thus, if offspring quality is correlated to maternal condition, mothers in good condition would benefit more from producing a son. In birds, the female is the heterogametic sex (ZW), giving her the potential control over whether the gamete to be fertilized will be a male or a female. Several steps during ovum maturation have been proposed as crucial in avian embryo sex determination and potentially subject to manipulation (reviewed in Krackow 1995; Pike and Petrie 2003; Alonso-Alvarez 2006).

Maternal hormones are promising pathways by which environmental (e.g., season) and physiological (e.g., maternal condition) cues could be translated to the oocyte, thereby possibly influencing the process of sex determination to produce the sex that will most likely yield higher fitness returns in the prevailing environment. So far, in birds, maternal progesterone, corticosterone, and testosterone have been shown to be correlative and causally linked to skewed primary offspring sex ratio (Veiga et al. 2004; Correa et al. 2005; Pike and Petrie 2005, 2006; Rutkowska and Cichon 2006; Bonier et al. 2007; Goerlich et al. 2009; M. Müller, V. C. Goerlich, Y. Roelofs, C. Dijkstra,

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and T. G. G. Groothuis, unpublished manuscript). We will focus on the latter two hormones in this study.

Experimental elevation of maternal testosterone induced an overproduction of male embryos in zebra finches (*Taeniopygia guttata*; Rutkowska and Cichon 2006) and spotless starlings (*Sturnus unicolor*; Veiga et al. 2004), and similarly, our previous study indicated that female homing pigeons (*Columba livia domestica*) produce more male embryos after receiving testosterone (Goerlich et al. 2009). No effect of testosterone was observed in Japanese quail (*Coturnix coturnix japonica*; Pike and Petrie 2006), whereas corticosterone treatment resulted in a female-biased primary offspring sex ratio in that same study. Also white-crowned sparrows (*Zonotrichia leucophrys*; Bonier et al. 2007) and again homing pigeons (M. Müller, V. C. Goerlich, Y. Roelofs, C. Dijkstra, and T. G. G. Groothuis, unpublished manuscript) produced more female embryos after receiving corticosterone implants, whereas in European starlings (*Sturnus vulgaris*), not the primary but the secondary brood sex ratio was female biased (Love et al. 2005). It is worth noting here that the bioavailability of both testosterone and corticosterone is regulated by the same plasma-binding globulins (CBGs) in the circulation. Manipulation of either hormone might affect the competition for available binding sites, thereby changing the bioavailability independent of changes in CBG levels. Just as well, changes in testosterone or corticosterone might induce changes in CBG levels, thereby affecting the fraction of bound and free hormone, without visible changes in total hormone concentrations (Breuner and Orchinik 2002; Swett and Breuner 2008). Overall testosterone and corticosterone usually show an inverse relationship and opposite effects on primary sex ratio, but the complex regulating system through CBGs might be the reason studies find opposing results on the direction of correlation (Roberts et al. 2007).

An unresolved matter is whether actual maternal plasma levels, or, rather, concentrations of maternal steroids in the egg yolk, are influencing offspring sex ratio. Yolk hormones potentially play a role in embryo sex determination through meiotic drive (Rutkowska and Badyaev 2008), that is, influencing chromosome segregation during meiosis I, so that the desired sex chromosome (Z to become a male, W to become a female) would be retained in the ovum. In homing pigeons, testosterone implantation elevated plasma testosterone levels, but total yolk testosterone concentrations did not differ between treatment groups or between male and female eggs, suggesting that elevation of maternal plasma levels directly induced the male-biased sex ratio (Goerlich et al. 2009). However, yolk is deposited in concentric layers, which have been shown to vary in their hormonal content (Lipar et al. 1999; Hackl et al. 2003; Rettenbacher et al. 2005). Because the embryonic disk is embedded in the outermost layer of the ovum, it might be only the immediate surrounding hormone levels influencing meiosis.

To test the predictions of Trivers and Willard (1973), a range of studies manipulated maternal body condition and found effects on primary offspring sex ratio (Nager et al. 1999; Clout et al. 2002; Pike 2005; Whittingham et al. 2005; Robertson et

al. 2006). Correlative data, however, often lack the link between maternal condition and offspring sex (Addison et al. 2008; Laucht et al. 2008). The timing of recording maternal condition is crucial but varies to a high degree between studies (Cameron 2004). Relating a single measurement of maternal condition to offspring sex might not be the most appropriate approach. Indeed, in feral horses, the change of maternal mass from pre- to postconception predicted offspring sex more precisely than condition measured at conception alone (Cameron et al. 1999; Cameron and Linklater 2007).

To describe and measure body condition, the majority of studies use the residuals of an individual's body mass corrected for a structural size component, usually tarsus length in birds, as a proxy. However, as an individual changes in mass, because of either improved food conditions or physiological adaptations, several blood parameters change as well (Jenni-Eiermann and Jenni 1994; Alonso-Alvarez et al. 2002). Glucose and cholesterol are both blood metabolites whose levels indicate an individual's condition and correlate with offspring sex ratio. Female yellow-legged gulls (*Larus cachinnans*) with high levels of plasma cholesterol produced male-biased broods (Alonso-Alvarez and Velando 2003). Studies on the relation between glucose and offspring sex were solely conducted in mammals; in mice, a negative change of plasma glucose correlated with the overproduction of daughters (Cameron et al. 2008), and in female field voles (*Microtus agrestis*), exhibiting high plasma glucose and testosterone levels produced more sons (Helle et al. 2008). We repeatedly measured these parameters in our experiment as well to test their connection to maternal condition and whether absolute values, or, rather, the change in levels over time, would predict primary offspring sex ratio in birds. We were also interested in the relation between these blood metabolites and the steroid hormones testosterone and corticosterone, which likewise have been linked to variation in individual body condition. Generally, good body condition is positively correlated to circulating levels of testosterone, whereas individuals in worse condition exhibit higher levels of corticosterone (Kitaysky et al. 1999; Perez-Rodriguez et al. 2006). Both plasma testosterone and maternal condition were positively correlated to primary sex ratio in peafowl (*Pavo cristatus*), while plasma corticosterone showed a negative relation (Pike and Petrie 2005). In female quail, manipulation of testosterone did not affect fecal levels of corticosterone, nor did corticosterone implantation affect fecal levels of testosterone (Pike and Petrie 2006). Thus, the causality of the suggested triangular relation between testosterone, corticosterone, and body condition needs further studies, as do the pathways by which these hormones exert their effects on offspring sex ratio.

As a model species we used the homing pigeon, a nonseasonal breeder with a modal clutch size of two eggs, laid 44 h apart (Pike 2005; Goerlich et al. 2009). The readiness to breed and the regularity in oviposition make pigeons a suitable species to investigate mechanisms underlying offspring sex manipulation. But most important, so far three independent experimental studies have shown pigeon mothers to shift their primary offspring sex ratio, especially of the first egg, in response

to manipulation of maternal body condition (a decrease inducing a female-biased primary sex ratio; Pike 2005), testosterone (inducing a male bias; Goerlich et al. 2009), and corticosterone (inducing a female bias; M. Müller, V. C. Goerlich, Y. Roelofs, C. Dijkstra, and T. G. G. Groothuis, unpublished manuscript).

Our experimental design and treatment were basically repeats of our previous study (Goerlich et al. 2009) but included crucial additional measurements. We first investigated under unmanipulated standard settings whether maternal body condition and blood plasma concentrations of testosterone, corticosterone, cholesterol, and glucose predict embryo sex of first eggs. Subsequently, we treated the same females with testosterone, asking whether the elevation of testosterone directly affected embryo sex, or, rather, asserts its effects through influencing circulating levels of corticosterone, cholesterol, or glucose. Having females produce a baseline and a treatment clutch gave us the opportunity to make inter- and intraindividual comparisons to test the effects of testosterone elevation on these parameters. We conducted the experiment in two successive blocks with two different groups of females. The females used in the first block arrived at our laboratory in poor body condition. By giving birds the possibility to feed ad lib., we were able to test the suggestion of Cameron and Linklater (2007), whether the change in maternal body condition would predict primary offspring sex. Furthermore, after females had received testosterone, we measured testosterone concentrations in the outer yolk layer to explore treatment or sex-related variation and to test whether embryo sex is more likely to be affected by maternal plasma or yolk hormones.

Material and Methods

Experimental Design

The experimental setup consisted of two successive blocks of 30 breeding pairs; in both blocks, pairs produced a preimplantation first clutch and a postimplantation second clutch (see Fig. 1). In both blocks, females were killed after the first

egg of the second clutch, while males from the first block were reused in the second block.

Preceding the experiments, we housed the birds, which we obtained from several Dutch breeders, in unisex outdoor aviaries. In the experimental phases, we kept each breeding pair in single cages within one room and provided ad lib. food and water. Treatment alternated over the cages, and females were distributed evenly across the experimental groups based on age. Birds had auditory contact to all and visual contact to some except neighboring pairs. Light was set constant at a 14L : 10D cycle; room temperature was constant at 22°C (see Goerlich et al. 2009 for more details).

During both clutches, we blood-sampled females during the phase of rapid yolk deposition, which lasts about 6.5 d (Fig. 1; Birrenkott et al. 1988). We expected maternal hormone and metabolite levels at this time point to be adequate predictors of offspring sex and yolk hormone levels. All birds were sampled in one session, and for each individual, we noted the time between entering the room and retrieval of the sample. After measuring glucose concentrations, the fresh blood samples were centrifuged and the plasma was frozen at -20°C until hormone analysis (see "Hormone Analysis"). While the lights were on, the nests were checked once per hour to ascertain timing of oviposition. After removal of the first egg during clutch 1, pairs received dummy eggs to ensure clutch completion, after which the male was moved to an outdoor aviary to await the next experimental clutch. When all pairs had completed their first clutches, we implanted females subcutaneously with Silastic tubes (Raumedic, inner diameter 1 mm, outer diameter 3 mm) of 14 mm length, filled with crystalline testosterone (Sigma Chemical, St. Louis, MO), or empty sham implants (see Goerlich et al. 2009 for more details). Tubes were sealed at both ends with silicon glue; therefore, the actual portion filled approximated 10 mm testosterone. Three days after implantation, each female was paired with the same male as during the first clutch. During clutch 2, directly after oviposition of the first egg, females were killed using CO_2 and then stored overnight at 10°C . The next day females were measured (tarsus 0.1 mm),

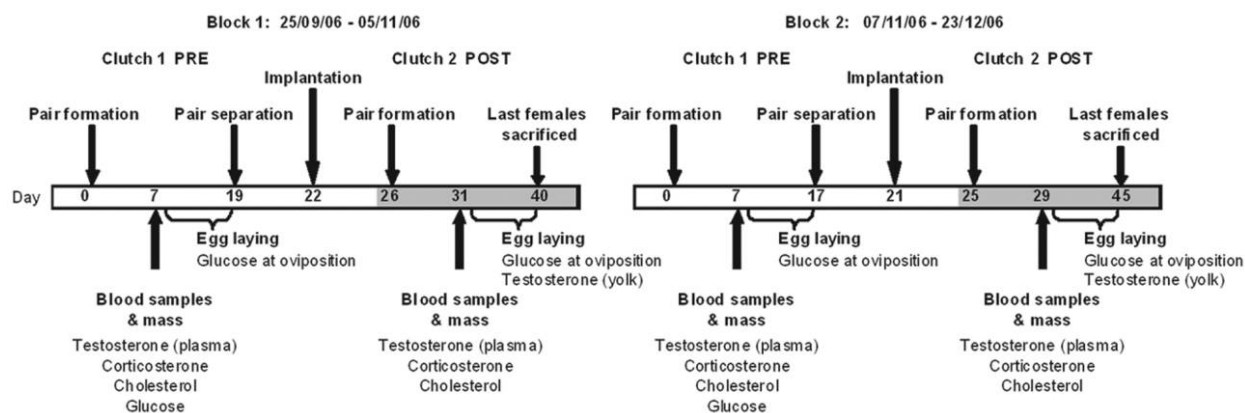


Figure 1. Experimental time line and sampling schedule.

Table 1: Results of generalized linear mixed models (GLMMs) combining different data subsets to predict sex of first eggs during the first preimplantation clutch

GLMM	<i>B</i>	SE	Wald χ^2_1	<i>P</i>
A. <i>N</i> = 16:				
Plasma testosterone	1.59	4.78	.11	.74
Plasma corticosterone	.80	2.00	.16	.69
Residual body mass	.08	.06	2.20	.14
B. <i>N</i> = 22:				
Plasma testosterone	1.06	2.48	.18	.67
Plasma cholesterol	−1.64	.97	2.87	.09
Residual body mass	.12	.06	3.27	.07
C. <i>N</i> = 27:				
Plasma corticosterone	.50	.89	.32	.57
Plasma cholesterol	−1.08	.59	3.37	.07
Residual body mass	.02	.02	2.24	.14
D. <i>N</i> = 27:				
Blood glucose	−.06	.22	.07	.79
Residual body mass	.01	.01	.48	.49

and oviducts and ovaries were removed to measure follicles for a concurrent study on ovarian dynamics (Goerlich et al. 2010). We retrieved the implants except from two testosterone-treated females. Since we could not ensure that the implants stayed in place after implantation, we excluded these birds from the clutch 2 analyses.

During both clutches, freshly laid eggs were placed into an incubator at 38°C for 72 h. This period ensures sufficient embryonic tissue for DNA sex determination while avoiding major changes in yolk hormone levels because of embryo metabolism (Gilbert et al. 2007). Following incubation, the eggs were frozen at −20°C for further analyses.

The second block mimicked the same experimental protocol except that females started the first clutch in a better body condition. Males were reused during the second block; however, they were assigned to the other treatment group and another cage. All experimental procedures were carried out under approval of the animal experimentation committee of the University of Groningen (license DEC 4347D).

Molecular Sexing

Based on the protocol used by Goerlich et al. (2009), we weighed albumen and yolk to the nearest 0.001 g, and yolks were frozen until hormone analysis (see “Hormone Analysis”). Embryonic DNA was extracted with Chelex, and the two homologous genes CHD-W (females) and CHD-Z (males and females) were amplified by polymerase chain reaction (PCR) using the primers P2 and P8 (Walsh et al. 1991; Griffiths et al. 1998). Control samples of known females (two bands) and males (one band) were incorporated in every PCR and in every gel electrophoresis. Eggs that did not develop a visible embryo after 3 d of incubation were considered as not fertilized. We

failed to determine the sex of one egg in the preimplantation testosterone group.

Hormone Analysis

Testosterone and Corticosterone Extraction. After separation of the yolk and albumen, we took a small subsample (ca. 100 mg) of the outermost yolk layer (layers still visible after 3 d of incubation). In all extractions, we incorporated control plasma or yolk pools to calculate interextraction variation. In brief, we added ca. 5,000 cpm radioactive-labeled testosterone (PerkinElmer Life and Analytical Sciences BV) to all samples to calculate hormone recovery. Testosterone plasma samples were extracted once with 2.5 mL of diethyl ether/petroleum benzene, 70 : 30 (vol/vol), followed by a single extraction with 1 mL 70% methanol. After drying under a nitrogen stream, the sample pellets were resuspended in phosphate-buffered saline with 1% gelatine (PBSG) 1 : 1 according to the original volume of the plasma sample. The yolk subsamples were treated similarly; only the diethyl ether/petroleum benzene extraction step was repeated once (Goerlich et al. 2009).

Corticosterone extraction and assay procedure were based on the steroid hormone assay protocol (Max Planck Institute of Ornithology, Andechs, Germany; Goymann et al. 2006). To calculate recoveries, we added ca. 2,500 cpm radioactive-labeled corticosterone (PerkinElmer Life and Analytical Sciences BV) to all samples. We extracted plasma samples twice with dichloromethane and, similar to the testosterone extraction, once with 70% methanol. After drying, the pellets were dissolved in PBSG. Average recoveries for plasma testosterone were 65% (range = 49%–78%), plasma corticosterone 80% (61%–99%), and outer yolk layer 74% (57%–83%).

Radioimmunoassay. Testosterone concentrations in plasma and yolk samples were measured using a commercial radioimmunoassay (RIA) kit (Active testosterone coated-tube RIA DSL-4000 kit, Diagnostic Systems Laboratories) with a detection limit of 0.02 ng/mL. We incorporated assay controls and standard curves in duplicates, the serial dilution reaching from 20 to 0.078 ng/mL. To perform the corticosterone RIA, we added antiserum (B3–163, Endocrine Sciences) and tritiated corticosterone to all samples and standard curves. After 30 min incubation, we separated bound and free fractions by adding dextran-coated charcoal, centrifuging 14 min thereafter, and decanting the supernatant into scintillation vials. Detection limit was 0.01 ng/mL. Corticosterone concentrations of some samples fell below detection limit and were substituted with 0.01 ng/mL for calculations. RIA concentrations were corrected for initial plasma volume/yolk mass and calculated as picogram per milligram of yolk and nanogram per milliliter of plasma, respectively. We calculated the coefficients of variation (CVs) based on assay controls and standard curves. Average interassay CVs were 6.4% (testosterone) and 12.1% (corticosterone), and average intra-assay CVs were 3.1% (testosterone) and 6.5% (corticosterone).

Table 2: Maternal parameters and generalized linear mixed model (GLMM) results

Parameter	Absolute Levels ^a				GLMM Results (Treatment × Clutch No.) ^b			
	Clutch 1		Clutch 2		<i>B</i>	SE	Wald χ^2	<i>P</i>
	Control	Testosterone	Control	Testosterone				
Plasma testosterone (ng/mL)	1.7 ± .4 (16)	1.8 ± .5 (15)	2.7 ± .4 (28)	3.1 ± .3 (26)	.274	.138	3.95	.047
Plasma corticosterone (ng/mL)	1.4 ± .4 (18)	1.1 ± .3 (14)	1.3 ± .5 (14)	1.1 ± .2 (15)	−.087	.263	.108	.74
Plasma cholesterol (mmol/L)	8.7 ± .3 (27)	8.9 ± .3 (26)	9.1 ± .4 (28)	9.9 ± .3 (27)	.735	.461	2.547	.11
Blood glucose (mmol/L) ^c	13.1 ± .3 (26)	13.1 ± .3 (25)	14.7 ± .2 (14)	14.2 ± .4 (19)	−.642	.514	1.565	.21
Residual body mass	−5.6 ± 9.7 (28)	−15.7 ± 5.8 (26)	13.8 ± 8.0 (29)	−8.3 ± 5.2 (26)	−4.403	9.484	.216	.64

Note. Values are presented ± SE. Sample sizes are included in parentheses. Significant results are depicted in bold.

^a Concentrations of maternal plasma hormones and metabolites during the pre- and postimplantation of clutches 1 and 2.

^b Results of GLMM testing the effects of treatment × clutch number on maternal plasma parameters and residual body mass.

^c Measured after oviposition of first egg.

Blood Glucose and Plasma Cholesterol

Glucose concentration (mmol/L) was measured directly after blood sampling in whole blood by applying a small drop of blood (ca. 5 μ L) to commercial strips (OneTouch Ultra, LifeScan; detection limit 1.1 mmol/L, mean CV = 2.1%). Plasma cholesterol was quantified using NobiCal (HITADO Diagnostic Systems), a commercial kit based on photometrical measurement, with a detection limit of 0.08 mmol/L (mean CV = 0.95%).

Plasma testosterone, corticosterone, cholesterol, and blood glucose levels were analyzed in a subset of animals, equally distributed across blocks, experimental groups, and both embryo sexes. Sample sizes are given throughout the text and in tables.

Statistical Analyses

We restricted our analyses to first eggs since these especially showed biases in primary sex ratio in our previous and other studies on pigeons (Pike 2005; Goerlich et al. 2009; Dijkstra et al., forthcoming; M. Müller, V. C. Goerlich, Y. Roelofs, C. Dijkstra, and T. G. G. Groothuis, unpublished manuscript). We fitted generalized linear mixed models (GLMMs) with hierarchical levels (MlwiN 2.02), nesting clutch number within female ID within block to control for our experimental design. The significance of variables was tested using the Wald statistic, which follows a χ^2 distribution. Nonsignificant interactions were removed in the model following the backward stepwise method (Grafen and Hails 2002). Models that did not require a hierarchical structure were calculated using SPSS (ver. 16.0).

We approximated female body condition by calculating the unstandardized residuals from a mixed model with female body mass as the dependent (four measurements taken at consecutive time points during the experimental clutches; Fig. 1), tarsus as the predictor, and female ID as a random factor ($N = 220$, Wald $\chi^2 = 9.572$, $P < 0.0001$).

Yolk and plasma testosterone, as well as plasma corticosterone, were log transformed to achieve an overall normal dis-

tribution. Hormone profiles are known to vary with the stage of the ovulation cycle (Alonso-Alvarez and Velando 2003; Jensen and Durrant 2006). Plasma testosterone was significantly affected by the reproductive stage of the female at blood sampling, such that levels were higher shortly before oviposition; thus, levels varied negatively with the interval (d) between blood sampling and oviposition ($B = -0.195$, $SE = 0.085$, Wald $\chi^2 = 5.294$, $P = 0.02$).

Plasma corticosterone levels rose significantly with the time between entering the animal room and taking the blood sample (mean = 271 s, $SD = \pm 119$, range = 70–561; $B = 0.002$, $SE = 0.001$, Wald $\chi^2 = 9.572$, $P = 0.002$). To account for variation in relation to the reproductive stage and time of sample retrieval, we incorporated both the sampling time and the interval between blood sampling and oviposition in all our models.

Sex of first egg embryos (0 representing female, 1 representing male) was transformed with the logit link function for binary data (Rasbash et al. 2005). We first analyzed the relation between the measured parameters and embryo sex of first eggs during the first preimplantation clutch by testing each parameter separately, thus with plasma testosterone, corticosterone, cholesterol, glucose, or residual mass as predictor. To further explore the relation of the latter parameters to one another and first egg sexes, we performed GLMMs, testing several predictor variables in one model. Because we did not have all measurements of all females, we calculated four separate models to retain sufficient data points in each model in relation to the predictor (see Table 1). Other subsets did not provide enough data to properly estimate the model.

To test effects of testosterone treatment and analyze inter- and intraindividual changes, we used the first preimplantation clutch as the within-female control for the second post-implantation clutch. We fitted models with clutch number, female ID, and block as levels and the interaction of treatment × clutch number as predictor of embryo sex of first eggs, plasma hormones, metabolites, or residual mass during both clutches. Subsequently, we incorporated block, not as level

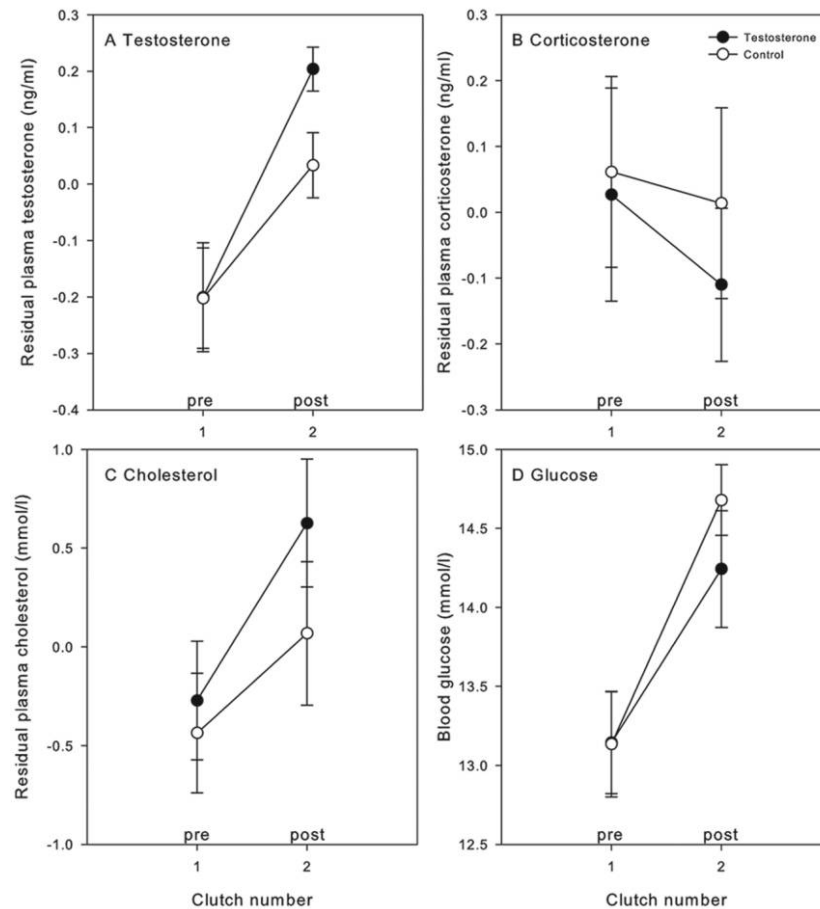


Figure 2. Effect of testosterone implantation on (A) plasma testosterone, (B) corticosterone, (C) cholesterol during the phase of rapid yolk deposition, and (D) blood glucose at oviposition, comparing both pre- and postimplantation clutches 1 and 2. Depicted are the residuals of the linear regression between the plasma parameter and the interval between blood sampling and oviposition (and time until sampling for corticosterone). See text for statistics. Graphs represent mean \pm SE.

but as categorical predictor to investigate differences between the successive experimental blocks. Log yolk testosterone concentrations were analyzed with a two-level model (female ID and block), with treatment, embryo sex, and their interaction as predictors.

On the basis of Cameron and Linklater (2007) and Cameron et al. (2008), we calculated change in female mass, log testosterone, log corticosterone, cholesterol, and glucose to predict the change in sex of the first egg from the first clutch to the second clutch. From 37 females, we collected a fertilized first egg during both clutches, and we divided the change in sex of first eggs in four compositions: (1) first clutch daughter \rightarrow second clutch son, (2) first daughter \rightarrow second daughter, (3) first son \rightarrow second son, and (4) first son \rightarrow second daughter. This way, we made our analyses sensitive for individual propensities of females to produce a certain sex. We then applied multinomial regressions with change in female mass or the respective plasma parameter as the predicting variable and the four compositions as unordered categorical dependents, with

first son \rightarrow second daughter as a reference category. In all tests, significance levels were set to $\alpha < 0.05$ (two tailed), and graphs represent means \pm SEs.

Results

Preimplantation Clutch 1

None of the measured maternal plasma parameters or residual body mass predicted sex of first eggs, either when tested separately (testosterone, $N = 24$, $B = -0.88$, $SE = 1.63$, Wald $\chi^2_1 = 0.29$, $P = 0.59$; corticosterone, $N = 27$, $B = -0.03$, $SE = 0.71$, Wald $\chi^2_1 = 0.001$, $P = 0.97$; cholesterol, $N = 43$, $B = -0.19$, $SE = 0.23$, Wald $\chi^2_1 = 0.68$, $P = 0.41$; glucose, $N = 27$, $B = -0.05$, $SE = 0.23$, Wald $\chi^2_1 = 0.04$, $P = 0.84$; residual body mass, $N = 44$, $B = 0.01$, $SE = 0.01$, Wald $\chi^2_1 = 1.35$, $P = 0.25$) or when included into one GLMM (Table 1). In Table 1, parts B and C, both cholesterol and residual body mass showed a trend toward significance, with high cholesterol associated with more female first egg sexes and high residual

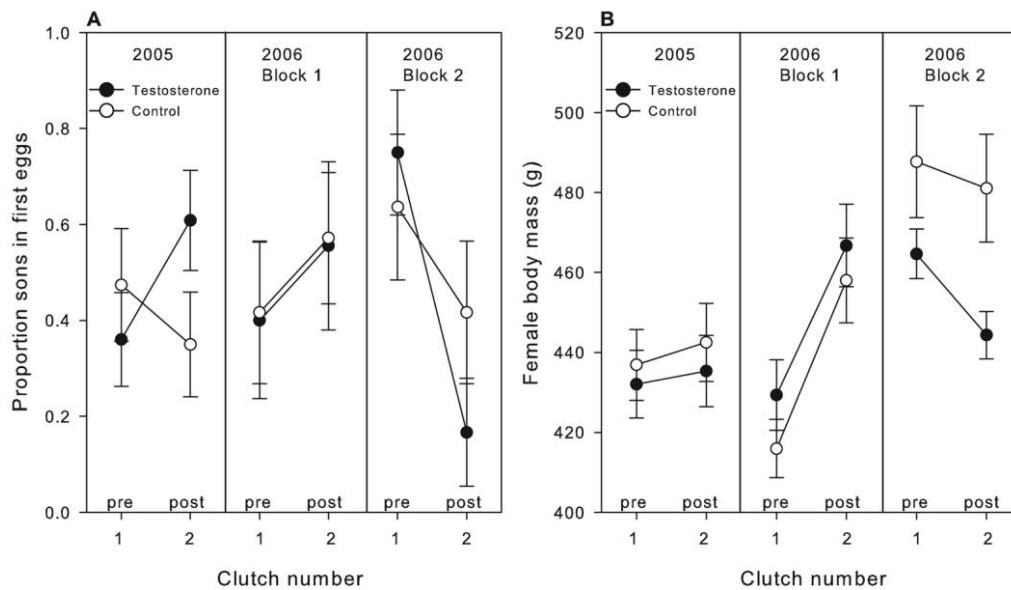


Figure 3. A, Sex ratio of first eggs comparing both pre- and postimplantation clutches 1 and 2 during the previous experiment (2005) and the two successive blocks of this experiment (2006). B, Female body mass comparing both pre- and postimplantation clutches 1 and 2 during the previous experiment (2005) and the two successive blocks of this experiment (2006). Graphs represent mean \pm SE.

mass associated with more male first egg sexes (Table 1); the signs of the slopes were the same as when the parameters were tested alone. In neither model did sampling time or interval between blood sampling and oviposition reach significance (all $P > 0.09$).

The Effects of Testosterone Implantation

To analyze the effects of testosterone treatment within females and between treatment groups, we tested the interaction of treatment \times clutch number on residual mass, plasma concentrations of testosterone, corticosterone, and cholesterol (measured during the phase of rapid yolk deposition; Fig. 1) and blood glucose (measured on an individual level after oviposition; Fig. 1). Because the variables were corrected in the statistical models, we listed absolute concentrations of all plasma parameters as well (see "Absolute Levels" in Table 2). Compared with the preimplantation levels and the controls, testosterone treatment significantly elevated plasma testosterone within the physiological range, but none of the other parameters were affected (Table 2, "GLMM Results"; Fig. 2).

On the basis of a similar experiment in 2005 (Fig. 2A; Goerlich et al. 2009), we predicted first eggs of testosterone-manipulated females to contain more sons compared with sham-implanted controls. Surprisingly, in this experiment, primary sex ratio did not differ between treatment groups (treatment \times clutch number: Wald $\chi^2_1 = 1.242$, $P = 0.27$, $N = 92$). To validate the significance of our previous findings, we pooled the data sets from both testosterone experiments (2005 and 2006). The interaction between year \times treatment \times clutch number (Wald $\chi^2_1 = 4.108$, $P = 0.043$) indicated that the effect of testosterone treatment on primary sex ratio was sig-

nificantly different between the 2005 and 2006 experiments. The most likely underlying cause is analyzed below.

Block Effects on Female Body Mass and Primary Sex Ratio of First Eggs

Basically, setup and treatment were the same between the experiments; however, 2005 was conducted in one block, consisting of 58 pairs. In 2006, we set up two successive experimental blocks of 30 pairs each. Compared with females used in the second block, females in the first block started the experiment in relatively low body condition and gained weight during the course of the experimental clutches (Fig. 3B). In contrast, females of 2005 remained stable in mass across the two experimental clutches (Fig. 3B). On the basis of these circumstances, we examined the sex ratio data for block effects and the influence of change in maternal mass.

During the first block, primary sex ratio of first eggs changed toward a higher proportion of male embryos from clutch 1 to clutch 2, while it changed toward a lower proportion of male embryos during the second block, irrespective of treatment group (Fig. 3A; treatment \times block \times clutch number: Wald $\chi^2_1 = 2.316$, $P = 0.13$). The interaction of block \times clutch number significantly predicted primary sex ratio of first eggs (Fig. 3A; Wald $\chi^2_1 = 7.235$, $P = 0.007$). None of the plasma parameters showed this pattern (corticosterone: $P = 0.80$; cholesterol: $P = 0.69$; testosterone: $P = 0.38$; glucose (oviposition): $P = 0.62$), yet female body mass also changed significantly with block \times clutch number (Fig. 3B; Wald $\chi^2_1 = 108.682$, $P < 0.001$), again irrespective of treatment (Fig. 3B; treatment \times block \times clutch number: Wald $\chi^2_1 = 1.185$, $P = 0.28$). Following Cameron and Linklater (2007), we tested



Figure 4. Categories of embryo sex composition of first eggs from preimplantation clutch 1 and postimplantation clutch 2 in relation to change in female body mass.

whether the change in female mass from clutch 1 to clutch 2 predicted the change in sex of first eggs (four categories; Fig. 4), which it significantly did (Wald $\chi^2_3 = 9.81$, $P = 0.02$). Females that gained weight switched from a daughter to a son, whereas females that lost weight tended to switch from a son to a daughter. No trend in weight change was apparent in females that consistently produced sons or daughters (Fig. 4). Change in first egg sex was not related to change in the plasma parameters (Δ testosterone: $N = 24$, $P = 0.7$; Δ corticosterone: $N = 17$, $P = 0.8$; Δ cholesterol: $N = 36$, $P = 0.08$; Δ glucose: $N = 16$, $P = 0.08$). However, the sample size concerning change in glucose was only half of that in the body-mass-change model, and given the borderline significance, it is likely that a larger sample size would have rendered a significant P value.

Differences in the amount of infertile eggs between treatment groups were negligible, and testosterone did not negatively affect female fecundity or egg fertility (Table 3). Also, differences between blocks were small, and it is therefore unlikely that any biases in offspring sex ratio had arisen from mechanisms acting on the secondary sex ratio level, for example, sex-specific oocyte fertilization or sex-specific embryo mortality.

Testosterone Concentrations in the Outer Yolk Layer of First Eggs

We measured outer yolk layer testosterone concentrations during postimplantation clutch 2. We could not detect differences between testosterone and control eggs (Fig. 5; N control = 15, testosterone = 14; Wald $\chi^2_1 = 0.436$, $P = 0.51$), male and female eggs (Fig. 5; N control: male = 9, female = 5; testosterone: male = 6, female = 7; Wald $\chi^2_1 = 1.355$, $P = 0.24$), or egg sex \times treatment (Wald $\chi^2_1 = 0.397$, $P = 0.53$). In addition, actual levels of maternal plasma testosterone did not predict yolk testosterone concentrations in the outer layer (Wald $\chi^2_1 = 0.015$, $P = 0.90$) and did not change in maternal mass (Wald $\chi^2_1 = 0.033$, $P = 0.86$).

Discussion

Preimplantation Clutch 1

Despite evidence in the literature on the correlation of testosterone, corticosterone, cholesterol, glucose, or residual mass with primary sex ratio (Alonso-Alvarez and Velando 2003; Pike and Petrie 2005; reviewed in Pike and Petrie 2003; Alonso-Alvarez 2006), we could find only weak support for these relations. According to our expectations, residual mass was positively related to embryo sex, and thus to a higher proportion of sons, in all the models. Surprisingly, however, cholesterol tended to be associated with a higher proportion of female embryos when incorporating residual mass and testosterone or corticosterone into the analyses.

Different sampling regimes (pre- vs. post-egg laying) might explain the discrepancy in effects between our study and the previously mentioned studies. The lack of significant predictors might be because plasma hormone and metabolite concentrations are fluctuating during the course of egg production (Gayathri et al. 2004; Jensen and Durrant 2006). Our blood samples provide only a limited snapshot of these variations, thereby perhaps missing the time point crucial for sex determination.

Table 3: Number of first eggs produced during the pre- and postimplantation clutches 1 and 2, presented separately for the successive experimental blocks

	Control		Testosterone	
	Fertile	Infertile	Fertile	Infertile
Block 1:				
Clutch 1	12	3	10	3
Clutch 2	14	1	9	3
Block 2:				
Clutch 1	11	2	13	1
Clutch 2	12	2	12	3

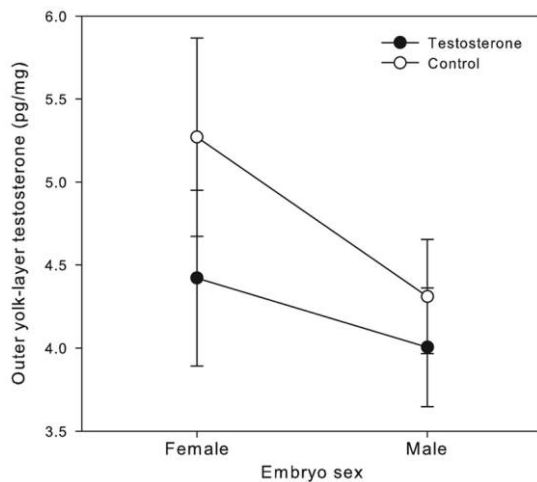


Figure 5. Testosterone concentrations in the outer yolk layer of first eggs, collected during postimplantation clutch 2, split for embryo sex. Graphs represent mean \pm SE.

The Effects of Testosterone Implantation

Testosterone implantation elevated plasma testosterone levels within a range comparable to the 2005 experiment, and therefore, we considered our treatment to be effective and a repeat of the previous study (Goerlich et al. 2009). However, testosterone treatment did not affect corticosterone levels or the other indexes related to maternal body condition. As mentioned previously, measurements of binding globulins would be useful to more accurately investigate the effects of testosterone on corticosterone.

Despite the elevated plasma testosterone levels, testosterone-treated females did not overproduce male embryos compared with control females, contradicting our earlier findings (Goerlich et al. 2009). The significant difference in treatment effects on primary sex ratio of first eggs between this study (2006) and the previous study (2005) strongly suggests that different factors were operating. As opposed to 2005, the females in 2006 underwent drastic changes in body mass between the experimental clutches, most probably because of differences in housing and feeding conditions. The change in body mass might have been a stronger cue in offspring sex adjustment and might have overridden the effects of elevated levels of maternal plasma testosterone. A positive change in maternal mass strongly predicted the change in sex of the first egg from a female embryo in clutch 1 to a male embryo in clutch 2. Interestingly, Cameron and Linklater (2007) showed that in feral horses the change in female mass from pre- to postconception is a much stronger predictor of offspring sex ratio than is a single measurement of maternal condition at the time of conception (Cameron et al. 1999). We also found a trend that change in glucose predicted change in primary sex ratio, also shown in mammals (Cameron et al. 2008). However, probably because of the small sample size, this effect did not reach full statistical significance.

Testosterone Concentrations in the Outer Yolk Layer of First Eggs

Regarding potential mechanisms underlying biased offspring sex ratio, meiotic drive has been suggested by several researchers (Howe 1977; Krackow 1995; Correa et al. 2005; Rutkowska and Badyaev 2008). It is not clear yet whether chromosome segregation during meiosis I could be affected by circulating maternal plasma hormones or by maternal hormones in the yolk. In case of the latter, we would expect to find sex-specific patterns in yolk hormone concentrations (this has been demonstrated in some species; Müller et al. 2002; Gilbert et al. 2005; Rutstein et al. 2005). In the homing pigeon, we did not detect sex-specific variation in total yolk testosterone (Goerlich et al. 2009) or in concentrations of the outer yolk layer (this study), which is immediately adjacent to the embryonic disk. In both studies, we analyzed eggs produced after mothers had received the testosterone or sham implants (clutch 2). However, in this study, testosterone did not affect the sex of first eggs, and although change in maternal mass was predictive of embryo sex of first eggs, it did not predict outer yolk layer testosterone. We cannot exclude the possibility that in our previous study, testosterone treatment elevated concentrations in the outer layer of the yolk. Therefore, we cannot reject the occurrence of meiotic drive because it might not be yolk testosterone levels directly influencing meiosis but another yolk hormone or metabolite influenced by our treatment (Goerlich et al. 2009), by changes in maternal mass (this study), or by a combination of these factors. Under these assumptions, it remains unclear why in homing pigeons meiotic drive should be restricted to first eggs.

Remarkably, despite elevated maternal plasma testosterone levels, total and outer yolk layer testosterone concentrations did not differ between treatment groups. Pigeons might be able to regulate yolk hormone concentrations independently from their own circulating levels.

Until now, four independent experimental studies on homing pigeons found mothers to bias the sex ratio of their first eggs: testosterone treatment (Goerlich et al. 2009) or a positive change in mass resulted in a male-biased sex ratio (this study), whereas under corticosterone treatment (M. Müller, V. C. Goerlich, Y. Roelofs, C. Dijkstra, and T. G. G. Groothuis, unpublished manuscript) or following negative changes in mass, females produced more daughters (Pike 2005; this study). Despite the lack of a causal link between testosterone, corticosterone, and residual body mass in this study, these factors frequently correlate under natural conditions. Our findings suggest that the mechanism of primary sex manipulation is affected not only by the absolute values but also by the complex interactions between maternal hormones and (indexes of) residual body mass.

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Literature Cited

- Addison B., A.S. Kitaysky, and J.M. Hipfner. 2008. Sex allocation in a monomorphic seabird with a single-egg clutch: test of the environment, mate quality, and female condition hypotheses. *Behav Ecol Sociobiol* 63:135–141.
- Alonso-Alvarez C. 2006. Manipulation of primary sex-ratio: an updated review. *Avian Poult Biol Rev* 17:1–20.
- Alonso-Alvarez C., M. Ferrer, and A. Velando. 2002. The plastic index of body condition in yellow-legged gulls *Larus cachinnans*: a food-controlled experiment. *Ibis* 144:147–149.
- Alonso-Alvarez C. and A. Velando. 2003. Female body condition and brood sex ratio in yellow-legged gulls *Larus cachinnans*. *Ibis* 145:220–226.
- Birrenkott G.P., M.A. Shoop, K. Cooper, and M. Wiggins. 1988. Ovarian follicular-growth and maturation in the domestic pigeon and Guinea fowl (*Numida meleagris*). *Poult Sci* 67: 1783–1786.
- Bonier F., P.R. Martin, and J.C. Wingfield. 2007. Maternal corticosteroids influence primary offspring sex ratio in a free-ranging passerine bird. *Behav Ecol* 18:1045–1050.
- Breuner C.W. and M. Orchinik. 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* 175:99–112.
- Cameron E.Z. 2004. Facultative adjustment of mammalian sex ratios in support of the Trivers–Willard hypothesis: evidence for a mechanism. *Proc R Soc B* 271:1723–1728.
- Cameron E.Z., P.R. Lemons, P.W. Bateman, and N.C. Bennett. 2008. Experimental alteration of litter sex ratios in a mammal. *Proc R Soc B* 275:323–327.
- Cameron E.Z. and W.L. Linklater. 2007. Extreme sex ratio variation in relation to change in condition around conception. *Biol Lett* 3:395–397.
- Cameron E.Z., W.L. Linklater, K.J. Stafford, and C.J. Veltman. 1999. Birth sex ratios relate to mare condition at conception in Kaimanawa horses. *Behav Ecol* 10:472–475.
- Clout M.N., G.P. Elliott, and B.C. Robertson. 2002. Effects of supplementary feeding on the offspring sex ratio of kakapo: a dilemma for the conservation of a polygynous parrot. *Biol Conserv* 107:13–18.
- Correa S.M., E. Adkins-Regan, and P.A. Johnson. 2005. High progesterone during avian meiosis biases sex ratios toward females. *Biol Lett* 1:215–218.
- Dijkstra C., B. Riedstra, A. Dekker, V.C. Goerlich, S. Daan, and T.G.G. Groothuis. Forthcoming. An adaptive annual rhythm in the sex of first pigeon eggs. *Behav Ecol Sociobiol*.
- Gayathri K.L., K.B. Shenoy, and S.N. Hegde. 2004. Blood profile of pigeons (*Columba livia*) during growth and breeding. *Comp Biochem Physiol A* 138:187–192.
- Gilbert L., E. Bulmer, K.E. Arnold, and J.A. Graves. 2007. Yolk androgens and embryo sex: maternal effects or confounding factors? *Horm Behav* 51:231–238.
- Gilbert L., A.N. Rutstein, N. Hazon, and J.A. Graves. 2005. Sex-biased investment in yolk androgens depends on female quality and laying order in zebra finches (*Taeniopygia guttata*). *Naturwissenschaften* 92:178–181.
- Goerlich V.C., C. Dijkstra, and T.G.G. Groothuis. 2010. Effects of *in vivo* testosterone manipulation on ovarian morphology, follicular development, and follicle yolk testosterone in the homing pigeon. *J Exp Zool* 311A:1–11.
- Goerlich V.C., C. Dijkstra, S.M. Schaafsma, and T.G.G. Groothuis. 2009. Testosterone has a long-term effect on primary sex ratio of first eggs in pigeons: in search of a mechanism. *Gen Comp Endocrinol* 163:184–192.
- Goymann W., M. Trappschuh, W. Jensen, and I. Schwabl. 2006. Low ambient temperature increases food intake and dropping production, leading to incorrect estimates of hormone metabolite concentrations in European stonechats. *Horm Behav* 49:644–653.
- Grafen A. and R. Hails. 2002. *Modern Statistics for the Life Sciences*. Oxford University Press, Oxford.
- Griffiths R., M.C. Double, K. Orr, and R.J.G. Dawson. 1998. A DNA test to sex most birds. *Mol Ecol* 7:1071–1075.
- Hackl R., V. Bromundt, J. Daisley, K. Kotrschal, and E. Mostl. 2003. Distribution and origin of steroid hormones in the yolk of Japanese quail eggs (*Coturnix coturnix japonica*). *J Comp Physiol B* 173:327–331.
- Helle S., T. Laaksonen, A. Adamsson, J. Paranko, and O. Huitu. 2008. Female field voles with high testosterone and glucose levels produce male-biased litters. *Anim Behav* 75:1031–1039.
- Howe H.F. 1977. Sex-ratio adjustment in common grackle. *Science* 198:744–746.
- Jenni-Eiermann S. and L. Jenni. 1994. Plasma metabolite levels predict individual body-mass changes in a small long-distance migrant, the garden-warbler. *Auk* 111:888–899.
- Jensen T. and B. Durrant. 2006. Assessment of reproductive status and ovulation in female brown kiwi (*Apteryx mantelli*) using fecal steroids and ovarian follicle size. *Zoo Biol* 25:25–34.
- Kitaysky A.S., J.C. Wingfield, and J.F. Piatt. 1999. Dynamics of food availability, body condition, and physiological stress response in breeding black-legged kittiwakes. *Funct Ecol* 13: 577–584.
- Krackow S. 1995. Potential mechanisms for sex-ratio adjustment in mammals and birds. *Biol Rev Camb Philos Soc* 70: 225–241.
- Laucht S., E.H. Duval, and B. Kempenaers. 2008. Maternal correlates of brood sex ratio variation in the lekking lance-tailed manakin *Chiroxiphia lanceolata*. *J Avian Biol* 39:198–205.
- Lipar J.L., E.D. Ketterson, V. Nolan, and J.M. Casto. 1999. Egg yolk layers vary in the concentration of steroid hormones in two avian species. *Gen Comp Endocrinol* 115:220–227.
- Love O.P., E.H. Chin, K.E. Wynne-Edwards, and T.D. Williams. 2005. Stress hormones: a link between maternal condition and sex-biased reproductive investment. *Am Nat* 166:751–766.

- Müller W., C.M. Eising, C. Dijkstra, and T.G.G. Groothuis. 2002. Sex differences in yolk hormones depend on maternal social status in leghorn chickens (*Gallus gallus domesticus*). *Proc R Soc B* 269:2249–2255.
- Nager R.G., P. Monaghan, R. Griffiths, D.C. Houston, and R. Dawson. 1999. Experimental demonstration that offspring sex ratio varies with maternal condition. *Proc Natl Acad Sci USA* 96:570–573.
- Perez-Rodriguez L., J. Blas, J. Vinuela, T.A. Marchant, and G.R. Bortolotti. 2006. Condition and androgen levels: are condition-dependent and testosterone-mediated traits two sides of the same coin? *Anim Behav* 72:97–103.
- Pike T.W. 2005. Sex ratio manipulation in response to maternal condition in pigeons: evidence for pre-ovulatory follicle selection. *Behav Ecol Sociobiol* 58:407–413.
- Pike T.W. and M. Petrie. 2003. Potential mechanisms of avian sex manipulation. *Biol Rev* 78:553–574.
- . 2005. Maternal body condition and plasma hormones affect offspring sex ratio in peafowl. *Anim Behav* 70:745–751.
- . 2006. Experimental evidence that corticosterone affects offspring sex ratios in quail. *Proc R Soc B* 273:1093–1098.
- Rasbash J., F. Steele, W. Browne, and B. Prosser. 2004. A User's Guide to MLwiN, Version 2.0. Centre for Multilevel Modelling, University of Bristol, Bristol.
- Rettenbacher S., E. Mostl, R. Hackl, and R. Palme. 2005. Corticosterone in chicken eggs. *Ann NY Acad Sci* 1046:193–203.
- Roberts M., K. Buchanan, D. Hasselquist, and M. Evans. 2007. Effects of testosterone and corticosterone on immunocompetence. *Horm Behav* 51:126–134.
- Robertson B.C., G.P. Elliott, D.K. Eason, M.N. Clout, and N.J. Gemell. 2006. Sex allocation theory aids species conservation. *Biol Lett* 2:229–231.
- Rutkowska J. and A.V. Badyaev. 2008. Review: meiotic drive and sex determination: molecular and cytological mechanisms of sex ratio adjustment in birds. *Philos Trans R Soc B* 363:1675–1686.
- Rutkowska J. and M. Cichon. 2006. Maternal testosterone affects the primary sex ratio and offspring survival in zebra finches. *Anim Behav* 71:1283–1288.
- Rutstein A.N., L. Gilbert, P.J.B. Slater, and J.A. Graves. 2005. Sex-specific patterns of yolk androgen allocation depend on maternal diet in the zebra finch. *Behav Ecol* 16:62–69.
- Swett M.B. and C.W. Breuner. 2008. Interaction of testosterone, corticosterone, and corticosterone binding globulin in the white-throated sparrow (*Zonotrichia albicollis*). *Comp Biochem Physiol A* 151:226–231.
- Trivers R.L. and D.E. Willard. 1973. Natural-selection of parental ability to vary sex-ratio of offspring. *Science* 179:90–92.
- Veiga J.P., J. Vinuela, P.J. Cordero, J.M. Aparicio, and V. Polo. 2004. Experimentally increased testosterone affects social rank and primary sex ratio in the spotless starling. *Horm Behav* 46:47–53.
- Walsh P.S., D.A. Metzger, and R. Higuchi. 1991. Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10:506–513.
- Whittingham L.A., P.O. Dunn, and J.K. Nooker. 2005. Maternal influences on brood sex ratios: an experimental study in tree swallows. *Proc R Soc B* 272:1775–1780.